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Comparative degradomics of porcine and human wound exudates unravels biomarker candidates for assessment of wound healing progression in trauma patients

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ABSTRACT

Impaired cutaneous wound healing is a major complication in elderly people and patients suffering from diabetes with raising rates in industrialized countries. Heterogeneity of clinical manifestations hampers effective molecular diagnostics and decisions for appropriate therapeutic regimens. Using a customized positional quantitative proteomics workflow, we have established a time-resolved proteome and N-terminome resource from wound exudates in a clinically relevant pig wound model that we exploited as robust template to interpret a heterogeneous dataset from patients undergoing the same wound treatment. With zyxin, IQGA1 and HtrA1, this analysis and validation by targeted proteomics identified differential abundances and proteolytic processing of proteins of epidermal and dermal origin as prospective biomarker candidates for assessment of critical turning points in wound progression. Thus, we demonstrate the possibility of using a fine-tuned animal wound model to bridge the translational gap as prerequisite for future extended clinical studies with large cohorts of individuals affected by healing impairments. Data are available via ProteomeXchange with identifier PXD006674.
INTRODUCTION

Elderly people as well as patients suffering from diabetes are frequently affected by impaired wound healing resulting in a profound health problem and economic burden (Menke et al., 2007). The goal of advanced wound therapies is to overcome factors preventing normal wound closure which typically leave the wound in a state of chronic inflammation (Sarabahi, 2012). Assessing the efficacy of these treatment regimens can be difficult however and an improved understanding of the processes involved is crucial to better guide therapeutic decisions and provide optimal care to individual patients. Proteomics has revolutionized the daunting quest for suitable surrogate biomarkers that indicate progress of wound healing and thus response to treatment (Lindley et al., 2016). Proteomics analyses comparing exudates from patients with difficult-to-heal ulcers to samples from individuals with normal healing wounds primarily identified quantitative differences in proteins related to inflammation and tissue destruction (Eming et al., 2010, Krisp et al., 2013). However, additional studies monitoring wound fluid proteomes in patients at multiple time points after injury are needed to assess wound progression and to devise new strategies for the development of point-of-care tests to aid therapy decisions within the scope of personalized medicine.

We have established a wound fluid proteomics workflow that combines efficient relative depletion of highly abundant proteins with enrichment for protein N termini by Terminal Amine Isotopic Labeling of Substrates (TAILS) (Kleifeld et al., 2010, Kleifeld et al., 2011, Prudova et al., 2010, Sabino et al., 2017, Sabino et al., 2015). TAILS inherently extends the analysis to proteolytic events, which are among the most prominent molecular determinants of wound chronicity (McCarty and Percival, 2013, Sabino and auf dem Keller, 2015). Here, we applied this workflow to record a dataset from trauma patients undergoing Negative Pressure Wound Therapy.
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That we analyzed with help of a NPWT pig model as statistically robust template. Time-resolved abundance clustering of proteins and N termini together with comparative pathway analysis, validation by targeted proteomics and localization in mouse wound tissue identified proteins of epithelial and mesenchymal origins as indicative biomarker candidates for wound progression in NPWT treated patients.

RESULTS

Experimental model to monitor wound progression in NPWT

To assess critical turning points in acute wound healing (Figure 1A), we used a pig NPWT wound model (Sabino et al., 2015), in which we collected four polyurethane foams from the same full-excisional back wound (ø 3 cm) that each had been left in the wound site for two days covering a time period of up to eight days after injury. This time frame discriminately resembled a major onset of granulation tissue formation and reepithelialization at days 6 and 8 after wounding as observed macroscopically in wounds upon foam removal and by histological analysis of related tissue sections (Figure 1B). Importantly, this model used the same type of foam dressings and very closely reflected the clinical NPWT regimen, from which we collected material for comparative assessment of acute wound progression in trauma patients (Figure 1C). Thereby, polyurethane foams are constantly soaked with wound exudates, but are concomitantly invaded by cellular infiltrates of immune, dermal and epidermal origin (Figure 1D) and thus contain extra- and intracellular protein content from the wound site, resembling a ‘liquid biopsy’ of wound tissue.

Comparative degradomics analysis of wound exudates from pigs and trauma patients

For degradomics analysis, we extracted wound fluids from the polyurethane foams, subjected
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them to dynamic range reduction by enrichment of low-abundance proteins with combinatorial peptide ligand libraries (CPLL) and analyzed exudates by 4plex-iTRAQ-TAILS (Figure 2A). To efficiently distinguish between changes in proteolytic activity and protein abundance, the proteome was quantified based on fully tryptic peptides, natural N termini and tryptic C termini, while neo-N termini were separately used for neo-N-terminome quantification (Figure 2A).

Foams from trauma patients were sampled according to clinical criteria, resulting in exudates from heterogeneous non-equidistant time points after wounding (Figure 2B; Supplemental Figure S1). Patient samples could be grouped based on overall length of NPWT treatment into a group of ‘fast healers’ (group 1; 7 patients) and a group of ‘slow healers’ (group 2; 3 patients). Sampling time points for the first group aligned with those from the pig model, allowing respective grouping and synchronized quantitative analyses, while samples from the second patient group were treated as test cases for relative retardation in acute wound healing. Thereby, we analyzed human wound exudates by multiplexed iTRAQ-TAILS in a pooled reference design.

We collected a dataset of a total of 1060 quantifiable proteins and 1667 neo-N termini from 492 proteins from porcine wound exudates (Figure 2C; Supplemental Tables S1 to S13) that all together corresponded to 1206 high confidence protein identifications. Around 60% (650/1060) of proteins and ~40% (695/1667) of neo-N termini were quantified in at least three out of five replicates, allowing for statistical evaluation of differential abundances between time points. Due to inherent heterogeneity, numbers of identified proteins and neo-N termini significantly differed between patients in the human dataset that in total yielded 1410 high confidence identifications on the proteome level and 9802 neo-N termini corresponding to 2020 proteins (Figure 2D; Supplemental Tables S14 to S38) and thus with 2667 total proteins represents the largest human wound fluid proteome reported to date. These proteins included 75% (696) of 930 proteins
identified in pig wound exudates that could be mapped to human orthologs, demonstrating a high similarity between samples from the animal model and from trauma patients. Notably, modes and applied pressure during NPWT were similar among patients and were not correlated with massive differences in extend of cell infiltrates into foams as suggested by assessment of proportion of secreted and cellular proteins in individual patient datasets (Supplemental Figure S1).

**Analysis of pig dataset as robust model for quantitative profiling of wound progression**

To establish a robust quantitative model for wound progression, we exploited iTRAQ-based relative quantification of proteins and neo-N termini in wound exudates from multiple time points after injury in the statistically valid pig model. By one-way ANOVA, we identified 416 proteins (Supplemental Table S39) and 371 neo-N termini (Supplemental Table S40) with significant changes in abundance over time as well as a subset of 188 neo-N termini (Supplemental Table S41) that significantly changed in relation to the abundance of the whole protein (cleavages).

Next, we used soft clustering (fuzzy c-means) to assign differentially abundant proteins, neo-N termini and cleavages to clusters of time-resolved abundance profiles (Figure 3A). Differentially abundant proteins and neo-N termini clustered very similarly, with high abundances at D2 (cluster 1), at D4 (cluster 2), D4 and D6 (cluster 3), continuous increase in abundances over time (cluster 4) and marked increase in abundances at D8 (cluster 5). Comparative Ingenuity® pathway enrichment analysis of these clusters (Figure 3B) revealed a prevalence of pathways associated with inflammation (ROS/NOS in macrophages, acute phase signaling, complement system) and immediate consequences of injury (coagulation system) in clusters comprising proteins with high abundances in the middle (D4, D6) (clusters 2 and 3) and of pathways associated with cell proliferation and migration (remodeling of epithelial adherens junctions) in clusters including
proteins and related neo-N termini with high abundances at the end (D8) of the monitored healing phase (clusters 4 and 5). Proteins assigned to proteolytic cleavages followed similar abundance profiles over time, but temporal distributions of enriched biological pathways considerably differed from analyses on the levels of proteome and neo-N-terminome. For example, members of the coagulation system were high in abundance at D2 and D4 (cluster 3) but differentially processed mostly at D8 (cluster 4). Concomitantly, cleavages assigned to proteins associated with epithelial dynamics (remodeling of epithelial adherens junctions) decreased in abundance over time (cluster 1), while related proteins followed a complementary profile with high abundances at D8 (cluster 5). This could result in stabilization of these structures in addition to their increased abundance as indicator of wound progression.

**Exploiting pig data as template for interpretation of heterogeneous patient samples**

The inherent heterogeneity of patient samples prohibited a meaningful statistical interpretation of the human dataset following a similar strategy as for the analysis of data from the pig model. However, the high similarity between both treatment regimens allowed us to exploit the pig dataset as template for extraction of temporal abundance profiles for wound progression from the patient data. To do so, we extracted proteins, neo-N termini and cleavages from data collected from group 1 patients (‘fast healers’) that had similar patterns of abundance over time for each cluster and level of analysis calculated from the porcine dataset (Figure 3C; Supplemental Tables S42 to S44). Validity of this approach was demonstrated by assignment of associated proteins to wound-related pathways (Figure 3D), which showed similar enrichment scores across clusters as in the analysis of pig data with highest correlation on the level of proteins. For instance, correlation was high for inflammatory processes reflected by pathways including ROS/NOS in macrophages and atherosclerosis signaling. These were assigned to cluster 3 with increase in
abundance of associated proteins early and decrease later after wounding, indicating resolution of inflammation, an important factor for wound progression. The most striking consistency between both analyses was the strong increase in abundance of proteins associated with ‘remodeling of epithelial adherens junctions’ at the end of the monitored healing period, indicating efficient reepithelialization as a hallmark of the onset of the proliferative phase of wound healing. In particular, these proteins included the focal adhesion protein zyxin (ZYX) and Ras GTPase-activating-like protein IQGAP1 (IQGA1) that both increased in abundance in wound exudates collected towards the end of NPWT treatment on the protein level (cluster 5), while abundances of products resulting from their proteolytic processing decreased over time (cluster 1).

Since N-terminal enrichment identifies proteins of low abundance that are not accessible by their tryptic peptides (auf dem Keller et al., 2013), we systematically mined clusters for neo-N termini, which had similar abundance profiles in the pig model and in group 1 patients and might be indicative for a critical turning point in wound progression. A prominent example was a neo-N terminus released from the high-temperature requirement A serine peptidase 1 (HtrA1) that was identified in both the pig model and in group 1 patients and that increased in abundance in wound fluids sampled at increasing time points after wounding (cluster 4). HtrA1 has been implicated in extracellular matrix remodeling (Grau et al., 2006), and thus its increased abundance might be associated with the onset of matrix production in the granulation tissue upon progression to the proliferative phase.

Validation of adherens junction components as markers of healing progression

To further quantitatively assess the correlation of time-resolved protein abundance profiles derived from the pig wound model and from patient data, we calculated a correlation matrix of normalized enrichment p-values for associated biological pathways (Figure 4A). This analysis
confirmed a very high correlation (0.84-0.98) of changes in abundance of proteins associated with dynamics of epithelial adherens junctions. These included ZYX and IQGA1 as well as multiple tubulin and actin isoforms that are all part of a protein network associated with epithelial cadherins and which all increased in abundance in wound exudates collected at the latest time point after injury in the pig model and at the end of NPWT in the group of ‘fast healers’ (Figure 4B). The same was true for HtrA1 whose differential abundance in wound exudates over time of healing was determined by iTRAQ-TAILS with help of a neo-N-terminal peptide close to the mature N terminus. However, HtrA1 could not be quantified at the protein level by this approach, since lysines are missing from the sequence of additionally identified fully tryptic peptides, preventing assessment of the detected neo-N terminus as potential product of a differential cleavage event. Therefore, we designed parallel reaction monitoring (PRM) assays to monitor differential abundances of HtrA1 on the protein level in samples from individual group 1 patients whose NPWT regimen was stopped at day 5 and 8, respectively, and from group 2 patients who were subjected to NPWT for up to 17 or even 25 days. These assays were further complemented by PRM assays for the quantitative assessment of ZYX and IQGA1 in the same set of wound exudates (Supplemental Figure S2; Supplemental Table S45). Indeed, all three proteins strongly increased in abundance in samples collected at the end of the NPWT compared to the prior sampling time point with exception of ZYX and IQGA1 in exudates from one of the group 1 patients. These results suggested the potential suitability of these proteins as markers of healing progression in wound exudates from NPWT treated patients. Most importantly, the established PRM assays were recorded under highly standardized conditions, which will allow direct transfer to extended studies with larger patient cohorts.
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**Assessment of abundance and localization of zyxin and HtrA1 in mouse wound tissue**

Our time-resolved degradomics analysis of wound fluids correlated increased abundances of zyxin and HtrA1 with healing progression in both the porcine wound model and in trauma patients. However, assessment of wound exudates does not provide spatial information on expression of these proteins in the wound. Therefore, we evaluated if zyxin and HtrA1 showed similar indicative changes in abundance during healing in a full-excisional mouse wound model.

We obtained tissue extracts from murine wound tissues at distinct time points after wounding (5-day wounds (dw), 14dw, 20dw, and unwounded skin) and assessed protein abundances by immunoblot analysis with antibodies specific for zyxin and HtrA1 (Figure 5). Indeed, we observed an increase in abundance of full length zyxin (~75 kDa) and of full length HtrA1 (~50 kDa) in protein lysates from 5dw compared to samples from normal skin and from wound tissue collected at later time points after wounding, supporting the observations made in the porcine model and in human patients. Moreover, we also detected a fragment of HtrA1 in lysates from 5dw with an expected molecular weight of the truncation observed by iTRAQ-TAILS (~48 kDa).

To obtain spatial information on the expression of zyxin and HtrA1 in wound tissue, we localized zyxin and HtrA1 in tissue sections of murine wounds by immunofluorescence and immunohistochemistry, respectively. Thereby, we detected an intense signal for zyxin in the hyperproliferative epithelium, whereas HtrA1 was expressed in the granulation tissue. Thus, these results suggest zyxin as a candidate marker for onset of reepithelialization and HtrA1 as an indicator of granulation tissue formation, which both are important processes at the critical turning point from the inflammatory to the proliferative phase of acute wound healing.
DISCUSSION

With ~3000 proteins identified in extracted wound fluids from both experimental systems, we significantly extended our knowledge of the proteome content of wound exudates. Our dataset represents a valuable resource for identification of key proteins during wound healing, design of pre-clinical studies and a framework for interpretation of clinical data. Moreover, we discriminated between changes in abundance of neo-N termini most likely due to variations in abundance of the substrate and changes most likely due to fluctuations in proteolytic activity. This to our knowledge previously unreported strategy was valuable for discriminative assessment of events associated with the coagulation system in the porcine model, where we observed increase in protein abundances mostly at D4 and D6 (cluster 3), but differential proteolysis at D8 (cluster 4). Hence, our method highlighted proteolytic events that may be important for wound healing progression. Extension of this method to other diseases associated with aberrant proteolytic activity (e.g. cancer, arthritis and inflammatory diseases) has the potential to identify key fluctuations of proteolytic activity during disease progression.

A major problem in current biomedical research is the translational gap, i.e. the failure in transferring results from basic research models to the clinic (Ansell et al., 2012). Direct analysis of samples from human clinical models often suffer from inherently high heterogeneity, while simple animal models might provide highly reproducible and homogeneous results, but often fail to sufficiently represent the human condition. We overcame this problem by using a porcine wound model that very closely resembled the clinical treatment regimen and thus allowed identification of events with high validity for the interpretation of the clinical dataset. Thereby, the high similarity between both models enabled even further advancing this concept by exploiting the homogenous pig dataset as template for modeling of human data. Thus, we were
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able to extract events from the human data with differential abundances between time points after wounding that would have evaded identification purely based on statistical filtering due to high variation within the dataset. This further strengthens the value of our refined porcine dataset as a reliable standard for proteomics and degradomics analysis of wound exudates from patients undergoing the widely applied NPWT regimen.

Healing impairments and chronic manifestation are heavily associated with imbalanced inflammation and tissue degradation (Menke et al., 2007). Therefore, targeted studies to identify indicative biomarkers for wound progression particularly in wound fluids focused on inflammatory mediators and tissue degrading proteases (Wiegand et al., 2010, Wysocki et al., 1993). Delayed or lack of onset of granulation tissue formation and reepithelialization are secondary to these alterations, but reflect the most critical turning points in healing advancement (Valenzuela-Silva et al., 2013). Indeed, in our study the most promising markers with highest correlation between pig model and patient data were directly related to migration and proliferation of epidermal cells (ZYX, IQGA1) and dermal fibroblasts (HtrA1) and thus primary indicators of these processes. While HtrA1 is a classically secreted protein (Zurawa-Janicka et al., 2017), both ZYX and IQGA1 are mainly associated with cytoskeletal structures and described to exert intracellular functions (Smith et al., 2014, Watanabe et al., 2015). Therefore, their identification might be restricted to exudates collected from NPWT foams with cellular infiltrates releasing high numbers of intracellular proteins. However, ZYX has been found in cellular secretomes (Molina et al., 2009) and thus might be released by alternative secretion or maybe present in exosomes. Moreover, ZYX was identified as a biomarker for non-small-cell lung cancer by targeted proteomics analysis of plasma samples (Kim et al., 2015), suggesting a general release of ZYX fragments into body fluids. Similarly, IQGA1 was identified by shotgun
proteomics in wound fluids collected from ulcer patients (Krisp et al., 2013), indicating its accessibility in wound exudates also by alternative sampling methods. Hence, although timely preceded by quantitative alterations of inflammatory mediators, the direct monitoring of proteins originating from epithelial or mesenchymal wound cells in wound fluids presents an alternative approach to more precisely capture the transition to the proliferative phase, especially when evaluating novel treatment regimens in therapy of stalled wounds.

Functionally, zyxin is related with signal transduction between focal adhesions and the nucleus, and it is described as the most prominent marker of mature focal adhesions (FA) (Wolfenson et al., 2013). Maturation of FA is induced by cellular migration and FA are believed to provide support for the forward protrusion of the leading edge in migrating cells (Bachir et al., 2014, Huttenlocher and Horwitz, 2011, Oakes and Gardel, 2014, Parsons et al., 2010, Ponti et al., 2004, Wolfenson et al., 2013). Our in vivo data suggesting the importance of zyxin during wound healing is in agreement with previous in vitro studies that describe a role of zyxin in efficient closure of scratched cell monolayers, since knock-down of zyxin in MDCK cells resulted in delayed scratch closure (Leccia et al., 1999, Nguyen et al., 2010). Another study showed that zyxin contributes to controlling the path of cell motion in three-dimensional matrixes, as opposed to other important FA components (e.g. talin and focal adhesion kinase) (Fraley et al., 2012). Thus, our analysis corroborates a functional role of zyxin in the proliferating wound epithelium, warranting further dissection of underlying mechanisms.

Demonstrating the increased sensitivity by selective enrichment of protein N termini in iTRAQ-TAILS, HtrA1 was identified by a neo-N terminus (RitrAQ-SAPLAAGCPDR) that increased in abundance at the end of NPWT treatment. However, PRM based quantification of HtrA1 in patient samples suggested that the cleavage was associated with synthesis of the protein and did
not represent a differential proteolytic event. Together with an arginine in P1 position, this indicates processing by a yet to be defined constitutively active protease of the trypsin-like S1 family, which would be in agreement with identification of the same neo-N terminus in human blood using an alternative N-terminomics approach (Wildes and Wells, 2010). We also showed increase in abundance of HtrA1 during wound healing in a murine model and that the protein mainly localized to the granulation tissue. Indeed, granulation tissue formation is among the most important events towards wound closure in humans (Valenzuela-Silva et al., 2013). HtrA1 can inhibit TGF-β signaling and cleave extracellular matrix proteins and proteoglycans, e.g. C-propeptides of fibril-forming types I, II and III procollagen (Murwantoko et al., 2004), fibronectin (Grau et al., 2006) and aggrecan (Launay et al., 2008). Moreover, HtrA1 has been described as possible tumor suppressor by inducing cell death through caspase-dependent or -independent apoptosis and anoikis (Skorko-Glonek et al., 2013). Thus, HtrA1 may have important functions in extracellular matrix remodeling, cellular proliferation and regulation of the inflammatory response during the proliferative phase of wound healing (Launay et al., 2008).

In conclusion, in this proof-of-principle study we exploited our robust analysis system for the time-resolved degradomics analysis of wound exudates (Sabino et al., 2017, Sabino et al., 2015) to identify novel indicative biomarker candidates for wound progression in trauma patients undergoing NPWT. It should be noted that results were recorded from a limited number of patients with considerable variabilities in wound type and location, but demonstrate the feasibility to bridge the translational gap to the clinic as prerequisite for more extended studies invoking large patient cohorts. For this, PRM targeted proteomics assays as exemplified in Figure 4 will be instrumental, since they will enable screening samples from high numbers of patients with highest reproducibility and sensitivity (Duarte and Spencer, 2016).
MATERIALS AND METHODS

Pig NPWT wound healing experiment

The porcine wound healing experiment was performed by BIOMATECH (France) on behalf of Paul Hartman AG (Germany) using a constant negative pressure of -125 mmHg as described previously (Sabino et al., 2015). Animals were housed in accordance with the EEC Directive 86/609, and all experiments were performed upon approval by an animal welfare committee (NAMSA/BIOMATECH 2009-12-04).

Collection of NPWT foam dressings containing human wound exudates

Foam dressings (VivanoMed, Hartmann, Heidenheim, Germany) from wounds of trauma patients were collected at the Department of Traumatology, Saarland University Medical Center (Homburg/Saar, Germany) upon approval by the local ethics committee (Ethics Committee at the Chamber of Physicians of the State of Saarland; approval no. Nr. 188/12). Following standard clinical procedures, foams were collected at each dressing change and stored frozen at -80 °C until extraction of wound exudates.

iTRAQ-TAILS analysis

Wound fluids were extracted from foam dressings, protein concentrations equalized using combinatorial peptide ligand libraries and analyzed by iTRAQ-TAILS following procedures detailed in (Sabino et al., 2015) and (Sabino et al., 2017).

PRM analysis

Wound fluid protein was processed following the iTRAQ-TAILS protocol but using a single iTRAQ reagent and without mixing of conditions. PRM assays were developed based on iTRAQ-TAILS shotgun analyses for ZYX (QNVAVNELC[+57]GR, m/z = 630.312, z = 2), IQGA1
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(ALQSPALGLR, m/z = 513.309, z = 2; EEIQSSISGVTAYNR, m/z = 863.926, z = 2; LTAEEEMDER, m/z = 547.245, z = 2; PHYGSVLDNER, m/z = 643.810 (429.542), z = 2(3)) and HtrA1 (GAC[+57.0]GQGQEDPNLSLR, m/z = 744.828, z = 2; LPVLLLGRR, m/z = 440.803, z = 2) and samples analyzed on a Q Exactive HF instrument (Thermo Scientific, Bremen, Germany). Skyline 3.5 (MacLean et al., 2010) and SpectroDive 7 (Biognosys, Zurich, Switzerland) were used for data analysis.

**Preparation of murine tissue extracts and immunoblot**

Wounds from C57BL/6 mice (Antsiferova et al., 2013) kindly provided by Dr. Maria Antsiferova (ETH Zurich) were homogenized with an ultra-turrax in T-PER buffer supplemented with cOmplete protease inhibitor cocktail (Roche, Mannheim, Germany) and analyzed by immunoblot using antibodies specific for zyxin (Santa Cruz Biotechnology (sc-15338), TX), HtrA1 (Abcam (ab199529), Cambridge, UK) or GAPDH (HyTest (5G4), Turku, Finland).

**Immunofluorescence and immunohistochemistry**

Wound sections (C57/BL6 mice (Telorack et al., 2016)) kindly provided by Dr. Matthias Schafer (ETH Zurich) were analyzed for zyxin and HtrA1 expression by immuno-fluorescence or -histochemistry using antibodies against zyxin B71 (Hoffman, 2003) (kindly provided by Prof. Mary Beckerle, University of Utah, USA) or HtrA1 (Abcam (ab199529), Cambridge, UK). Slides were stained with a Cy3-conjugated secondary antibody and Hoechst or using the Vectastain ABC system (Vector Laboratories, Burlingame, CA).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al., 2016) with the dataset identifier.
CONFLICT OF INTEREST

D. K. and H. S. are full-time employees of HARTMANN AG.

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FIGURE LEGENDS

Figure 1: Time-resolved sampling of wound exudates from pigs and NPWT patients. (a) Early phases of cutaneous wound healing. Onset of epidermal reepithelialization and formation of granulation tissue in the dermis mark a critical turning point in progression from the inflammatory to the proliferative phase. (b) Pig wound model. Exudates were collected from polyurethane foams that had been placed into wounds (ø 3cm) created on backs of animals and changed every two days after injury. Macroscopic images and histological analyses show formation of granulation tissue (arrows) and onset of reepithelization (dotted arrow). (c) Example of NPWT treated trauma wound with polyurethane foam in patient. (d) Schematic of NPWT system. Wound is filled with polyurethane foam, sealed with occlusive dressing and connected to a vacuum pump. Foams are constantly soaked with wound fluid and invaded by cellular infiltrates (foam histology), providing ‘liquid biopsies’ from multiple distinct time points after wounding.

Figure 2: Quantitative degradomics dataset of pig and human wound exudates. (a) iTRAQ-TAILS analysis. Wound exudates were eluted from foams, depleted from highly abundant proteins using combinatorial peptide ligand libraries (CPLL) and analyzed by 4plex-iTRAQ-TAILS. Proteins were quantified by tryptic peptides and natural termini (green) but independently of neo-N termini (red) to allow for identification of cleavage dynamics. Grey circles indicate iTRAQ labels. (b) Sample grouping and iTRAQ labeling scheme. Foams in pig model were sampled at days 2, 4, 6 and 8 after wounding. Patients were grouped into ‘fast healers’ and ‘slow healers’ with NPWT treatment times up to 25 days. For data analysis, sample time points from ‘fast healers’ were grouped and aligned with pig model to facilitate comparison. UpSet (Lex et al., 2014) visualization of iTRAQ-TAILS dataset from porcine (c) and human (d)
wound exudates. Numbers of identified proteins and neo-N termini per sample and overlaps are indicated. ‘Degree’ defines numbers of entries (cardinality) per level of intersecting sets. Bar graphs are scaled to the largest aggregate (‘A’ (e.g. Degree 5 for pig proteins)), ‘S’ defines the largest individual set (e.g. Pig5) and ‘U’ the overall size (Σ) of the dataset. Venn diagrams show overlap of proteins and neo-N termini on the level of protein within each species and overlap of proteins on the level of human orthologs between both datasets.

**Figure 3: Time-resolved abundance clustering and pathway enrichment.** (a) Abundance profiles of proteins, neo-N termini and cleavages in exudates from the pig NPWT model. Features with significantly differential abundance between time points after wounding were selected by one-way ANOVA (raw p-value<0.05) and assigned to abundance profiles by fuzzy c-means clustering. Selected features (ZYX, IQGA1, HTRA1) are highlighted. (b) Comparative biological pathway enrichment of clustered proteins, neo-N termini and cleavages from the pig wound model. Proteins assigned to each cluster were analyzed using Ingenuity for enriched canonical pathways, which were assessed across clusters by comparison analysis. Heatmaps were generated from enrichment p-values (Fisher’s exact test; -log_{10}(p)) normalized to an averaged value of zero and standard deviation of one across clusters. (c) Abundance profiles of proteins, neo-N termini and cleavages in exudates from NPWT trauma patients. Core members of each cluster (membership value >0.5 (proteins, neo-N termini), >0.4 (cleavages)) from the pig model were used to extract features with similar abundance profiles (Euclidian distance; ten closest features per template feature) from human data (fast healers). Selected features (ZYX, IQGA1, HTRA1) are highlighted. (d) Heatmaps of enrichment p-values from comparative canonical pathway analysis of human clusters calculated as described for the pig dataset.
Figure 4: Validation of epithelial adherens junctions components as indicators for wound progression. (a) Correlation of enriched pathways on the level of proteins between the pig and the human dataset. Correlation matrix was calculated for normalized pathway enrichment p-values across clusters on the level of proteins visualized in upper panels of Figures 3B and D. Color code and size of filled circles indicate a strong correlation between porcine wound exudates and samples from trauma patients in time-resolved abundance of epithelial adherens junctions components. (b) Abundance profiles of epithelial adherens junctions proteins in porcine and human wound exudates assessed by iTRAQ-TAILS. Fold changes (log2) of averaged abundance values in relation to the first sampling time point (D2) for samples from the pig model and trauma patients with fast healing kinetics show increase towards end of the monitored healing period. (c) PRM analysis of selected candidate proteins in patients with fast and slow healing kinetics. A general increase in abundance at the end of NPWT treatment indicates healing progression.

Figure 5: Expression and localization of zyxin and HtrA1 in mouse wounds. (a) Immunoblot and immunofluorescence analysis of zyxin expression in samples from a full-thickness excisional mouse wound model. Abundance of zyxin was highly increased in 5-day wounds (5dw) and staining restricted to the hyperproliferative epithelium (asterisk). Scale bar = 50 µm. (b) Immunoblot and immunohistochemistry analysis of HtrA1 expression. HtrA1 was highly abundant and proteolytically processed in lysates from 5-day wounds and staining was more prominent in granulation tissue (asterisk). Immunohistochemistry without anti-HtrA1 (2nd Ab ctrl) is shown to demonstrate specificity of HtrA1 staining. Scale bar = 100 µm.
adipose tissue
dermis
eschar
epidermis
hyper-proliferative epithelium
granulation tissue
wound fluid
foam
cellular infiltrate
a  zyxin
GAPDH

---75 kDa

b  HtrA1
GAPDH

--- 50 kDa

anti-zyxin 5dw

anti-HtrA1 5dw 2nd Ab ctrl 5dw